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Isolation of Leucogenenol from Bovine and Human Liver

BY F. A. H. RICE AND BADARUDDIN SHAIKH
Department of Chemistry, The American University, Washington, D.C. 20016, U.S.A.

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A compound has been isolated from both bovine and human liver that on intravenous or intraperitoneal injection into animals induces in 4–12h an increase in the number of peripheral neutrophils, and in 12–24h an increase in the number of peripheral lymphocytes. At 24h after injection there is also a two- to three-fold increase in the relative number of myeloblasts in the bone marrow. The procedure for isolation and the physical and chemical properties identify the compound as leucogenenol, isolated from the metabolic products of *Penicillium gilmanii* by Rice (1966).

Rice (1966) reported the isolation from the metabolic products of *Penicillium gilmanii* of a compound, leucogenenol, that induced a leukocytosis without a febrile response when it was injected intravenously or intraperitoneally into animals in quantities as low as 1μg/kg (Rice, 1968). It was also found that leucogenenol was not toxic when milligram quantities were injected into mice, rabbits, dogs or monkeys. Further studies suggested that leucogenenol stimulated the rate of formation, transformation or both of the series of cells leading to the mature neutrophil and also those cells leading to the mature lymphocyte (Rice & Darden, 1968). That leucogenenol stimulated the activity of the myeloid and lymphoid tissues was further demonstrated by studies on the regeneration of those tissues in sublethally irradiated mice (Rice, Lepick & Darden, 1968).

The above results suggested the possibility that leucogenenol functioned as a coenzyme or a portion of a coenzyme that was normally required for the growth, transformation or maturation of the progenitors of the peripheral blood cells. If this were the case leucogenenol could possibly occur normally in mammalian tissues. It was therefore decided to investigate this possibility, and it has now been found that leucogenenol is indeed normally present in bovine and human liver.

EXPERIMENTAL

Five different lots of freeze-dried aqueous extract of bovine liver were obtained in 2kg quantities from Mann Research Laboratories, New York, N.Y., U.S.A., and Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Human liver was obtained at autopsy, cut into cubes (side approx. 5cm), immediately frozen at solid-CO_2 temperature, evaporated to dryness from the frozen state and then ground with a mortar and pestle to a fine powder. Only human livers that showed no pathological findings were used. Either the powdered human liver or the commercially obtained liver powder (approx. 100g) was continuously extracted with methanol in a Soxhlet apparatus for 24h. The methanol extracts from 1kg of the dried liver powder were combined, evaporated to dryness under reduced pressure at 40–50°C (water-bath temperature) and extracted several times with portions (100ml) of diethyl ether and the ether extracts discarded. The residue was then dissolved in approx. 1litre of water, filtered through filter paper and then through approx. 50g of Amberlite 120 (H^+ form) contained in a fluted filter paper. The filtrate was immediately filtered through a column (5cm×54cm) of Amberlite 45A (OH^-form; 600g). The column was washed with approx. 20litres of water, eluted with aq.NH_3 (2%) and the subsequent procedure for the isolation of leucogenenol from the culture medium of *P. gilmanii* was followed in detail (Rice, 1966). No differences were found between leucogenenol from human or bovine liver and hence 'leucogenenol from liver' refers to leucogenenol isolated from either human or bovine liver. The yield was approx. 5mg.

Leucogenenol isolated from liver or from *P. gilmanii* showed single spots on t.l.c. on silica gel [no. 80.76; Research Specialities Co., Richmond, Calif., U.S.A.; zinc silicate (250mg/30g of gel) was added to produce a fluorescent adsorbent] when the slides were developed with: methanol–benzene (1:19, v/v), R_F 0.18; ethanol–benzene (1:19, v/v), R_F 0.28; or dioxan–benzene (1:9, v/v), R_F 0.66. No movement was detected when ethyl acetate, benzene or diethyl ether was used to develop the chromatogram. With ethanol or methanol the spot was so close to the solvent front that the determination of an R_F value was impossible. After development the spots were made visible either by examination under u.v. light or by spraying the slides with a saturated solution of 2,4-dinitrophenylhydrazine in 2M-HCl or by subjecting the slides to the action of I_2 vapour. Mixtures (1:1, w/w) of leucogenenol from liver with leucogenenol from *P. gilmanii* showed single spots with R_F values as given above.

G.l.c. was carried out on the trimethylsilyl derivative (Sweeley, Bently, Makita & Wells, 1963) with the F and

M model 700 laboratory chromatograph, manufactured by Hewlett–Packard, Avondale, Pa., U.S.A. It was equipped with model 5771A electrometer and dual flame detectors. A glass column (5ft coil, 2mm internal diameter) was packed with 3% (w/w) Ov-17 Gas Chrom Q80/100; injection temperature, was 285°C, column temperature 220°C and detector temperature 300°C; N_2 was used as the carrier gas with a flow rate of 40ml/min. Leucogenenol from either *P. gilmanii* or liver showed a single component with a retention time of approx. 3.9min with respect to diethyl ether. Mixtures (1:1, w/w) of leucogenenol from liver with leucogenenol from *P. gilmanii* showed a single component.

Optical rotations were measured on the Perkin–Elmer 141 spectrometer, with approx. 1ml of solution in a 10cm tube. Both leucogenenol from liver and leucogenenol from *P. gilmanii* had optical rotations in 0.02M-sodium borate of $[\alpha]_{589}^{20}$ 15° (*c* 0.9). The optical-rotatory-dispersion curves of leucogenenol from liver and from *P. gilmanii* in 0.02M-sodium borate were identical. The curves recorded on the ORD-UV-5 JASCO spectrometer (Durrum Instrument Corp., Palo Alto, Calif., U.S.A.) showed: $[\alpha]_{215}$ 92°; $[\alpha]_{222}$ 115°; $[\alpha]_{270}$ −26°; $[\alpha]_{320}$ −15°; $[\alpha]_{470}$ 0°; $[\alpha]_{620}$ 0° (*c* 0.9).

The i.r. spectra of leucogenenol obtained from either *P. gilmanii* or from liver were identical. The spectra measured on a Beckman model IR8 instrument showed major peaks at: 3279 (m); 3145 (sh); 2967 (s); 2907 (s); 2841 (sh); 1709 (s); 1634 (m); 1524 (w); 1427 (m); 1346 (m); 1252 (sh); 1196 (s); 1129 (m); 1075 (m); 1047 (sh); 1002 (w) cm^{-1}; where (m) means medium, (sh) shoulder, (s) strong and (w) weak.

The n.m.r. spectra of leucogenenol obtained from *P. gilmanii* or from liver were identical. The spectra measured with the Varian A-60 instrument in deuterated dimethyl sulphoxide with tetramethylsilane as the internal standard showed the following δ values (p.p.m.): 1.20 (3-H, doublet); 1.23 (3-H, doublet); 1.35 (1-H, complex); 1.95 (1-H, complex); 2.55 (2-H, multiplet); 3.60 (2-H); 3.70 (1-H, singlet); 4.00 (1-H, singlet; 4.10 (3-H, multiplet); 4.55 (broad band); 8.1 (1-H, singlet).

The u.v.-absorption spectra were measured on a Coleman–Hitachi spectrometer. The absorption spectrum of leucogenenol isolated from liver was identical with that obtained from *P. gilmanii*, reported by Rice & Barrow (1967).

Mass spectra were measured on the Hitachi–Perkin–Elmer RMU-66 mass spectrometer. No parent ion could be obtained with either leucogenenol or its trimethylsilyl derivative. However, a derivative, the monomethoxyacetate, prepared by treating leucogenenol with diazomethane followed by acetyl chloride in pyridine showed a parent ion at *m/e* 565. The spectra were the same for leucogenenol isolated from liver and from *P. gilmanii*. Copies of these mass spectra and of the n.m.r. spectra are available on request.

Six rabbits (New Zealand Whites, 2–3.5kg in weight) were each injected with 0.1μg of leucogenenol (isolated from human liver)/kg. Total and differential counts of the leucocytes in the peripheral blood were made at 2h intervals over a period of 24h. Results were the same as obtained with leucogenenol isolated from *P. gilmanii*, reported by Rice (1968). An increase in the number of neutrophils was found approx. 8h after injection, followed by an increase in the number of lymphocytes approx. 18h after injection.

Eighteen mice (Webster strain, 18–20g) were each injected with 4μg of leucogenenol. At intervals of 12, 24 and 48h groups of six animals were killed by cervical dislocation and smears made of small samples of their bone marrow. The smears were stained with Wright's stain and counterstained with Giemsa. Duplicate differential counts were made of 1000 cells each and the results averaged. Over a twofold increase in the relative number of myeloblasts was observed at the end of 12 and 24 h. Other bone-marrow cells also showed the same changes in distribution that were reported (Rice & Darden, 1968) to follow the injection of leucogenenol isolated from *P. gilmanii*.

DISCUSSION

The specificity of the procedure for the isolation of leucogenenol from *P. gilmanii*, involving as it does a separation of acidic constituents, solubility in various solvents and final chromatographic separation, strongly suggests that the compound isolated from bovine and human liver by the same procedure is leucogenenol.

That the compound isolated from liver is leucogenenol is also indicated by the fact that it has the same phyiscal properties as leucogenenol, such as identical u.v., i.r., n.m.r. and mass spectra and also the same biological properties, such as the ability to cause a leucocytosis in rabbits, characterized first by an increase in the number of neutrophils followed later by an increase in the number of lymphocytes in the peripheral blood, and also by its ability to cause a differential change in the type of cells found in the bone marrow. This last is especially marked by over a twofold increase in the relative numbers of myeloblasts in the bone marrow 24h after the injection of leucogenenol.

The finding that leucogenenol is a normal constituent of a mammalian tissue and the fact that it stimulates the formation of the blast forms of blood cells suggests that the compound plays a normal role in the regulation of the number of leucocytes and possibly erythrocytes in the body.

Although leucogenenol has been isolated from liver it need not originate in this organ. It could be produced at some other site and stored or metabolized in the liver.

It is noteworthy that leucogenenol is not chemically related to the neutropoetin isolated from serum by Komiya *et al.* (1959). Neutropoetin, although it also induces a leucocytosis, contains a number of amino acids none of which are found in leucogenenol. There is, in addition, no similarity in their physical properties.

The presence of leucogenenol in liver tissue raises the question as to why the ready availability of leucogenenol in liver does not, by getting into the bloodstream, cause a continuing increase in leucocytes in the body. At least two possibilities present

themselves: first, that the leucogenenol is bound to liver cells in a form that makes it unavailable and it is only liberated by the freezing, drying, grinding and alcohol extraction incidental to its isolation. Secondly, there is the possibility that somewhere in the animal's body other compounds are produced that regulate the availability of leucogenenol to the blood-cell-forming tissues.

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